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(57) Abstract

The invention relates to the discovery that nearly all medulloblastomas express neurotrophin-3 (NT-3) and its specific receptor TrkC. NT-3 was found to promote apoptosis in some medulloblastomas, therefore differing from the biologic actions most commonly described for neurotrophins. Based on these findings, the invention discloses methods of analyzing a medulloblastoma and for identifying molecules for treatment of medulloblastoma.

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NT-3 AND MEDULLOBLASTOMA

Statement as to Federally Sponsored Research

This invention was made in part with a grant from the National Institutes of Health. The Federal government has certain rights in the invention.

Background of the Invention

The invention is in the general field of neurotrophins.

Medulloblastomas are the most common malignant brain tumors of childhood, accounting for nearly 20% of primary central nervous system neoplasms that occur in children (Russell et al., Pathology of Tumours of the Nervous System. 5th ed. Baltimore: Williams and Wilkins, 1989, pp. 251-79). Since these cerebellar tumors tend to metastasize throughout the nervous system, therapy for children older than 3 years combines neurosurgical excision with external beam irradiation including the entire craniospinal axis (Hughes et al., *Cancer* 61:1992-8 (1988)) and multiple drug chemotherapy administered either for 3 - 4 months prior to radiation, or for up to 12 months afterward (Evans et al., *J. Neurosurg.* 72:572-82 (1990); Kretschmar et al., *J. Neurosurg.* 71:820-5 (1989); Packer et al., *J. Neurosurg.* 81:690-8 (1994)). Despite these measures, the prognosis of the tumor is not uniformly favorable. Sixty to eighty percent of children survive 5 years after diagnosis and treatment, and relapse infrequently thereafter (Packer et al., supra; Belza et al., *J. Neurosurg.* 75:575-582 (1991); Tarbell et al., *Cancer* 68:1600-4 (1991)). However, the remainder relapse and usually die despite receiving identical therapy.

Medulloblastomas most commonly express neurofilaments, neuron specific enolase, synaptophysin and nestin. Glial markers are expressed in a minority of cases. This indicates that most medulloblastomas are derived from neuronal progenitors in the developing cerebellum (Russell et al., supra; Kadin et al., *J. Neuropath. Exp. Neurol*. 29:583-600 (1970); Lendahl et al., *Cell* 60:585-95 (1990); Tohyama et al., *Am. J. Pathol*. 143:258-68 (1993); Trojanowski et al., *Mol. Chem. Neuropath*. 21:219-39 (1994); Trojanowski et al., *Mol. Chem. Neuropath*. 17:121-35 (1992)). Furthermore, 80 - 90% of the tumors express the transcription factors PAX6, EN2, and Zic, all of which are expressed only by developing cerebellar granule cells (Aruga et al., *J. Neurochem*. 63:1880-90 (1994); Kozmik et al., *Proc. Natl. Acad. Sci. USA* 92:5709-13 (1995); Yokota et al., *Cancer Res*. 56:377-83 (1996)).

The neurotrophins, including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), nerve growth factor (NGF), and neurotrophin 4/5 (NT-4/5) (Barde et al., *EMBO J*. 1:549-53; Berkemeier et al., *Neuron* 7:857-66 (1991); Ernfors et al., *Neuron* 5:511-26 (1990); Ip et al., *Cell* 47:545-54 (1986); Leibrock et al., *Nature* 341:149-52 (1989); Maisonpierre et al., *Science* 247:1446-51 (1990); Rosenthal et al., *Neuron* 4:767-73 (1990)), promote the expression of specific genes through activation of the Trks, a homologous family of specific receptor tyrosine kinases, with resulting biological effects on the nervous system. In this family, TrkA is associated with NGF (Kaplan et al., *Science* 252:554-7 (1991); Klein et al., *Cell* 65:189-97 (1991)), TrkB with BDNF and NT-4/5 (Berkemeier et al., supra), and TrkC with NT-3 (Lamballe et al., *Cell* 66:967-76 (19,91)). During their early development, granule cells preferentially express TrkB (Segal et al., *J. Neurosci*. 15:4970-81

(1995)). BDNF promotes the survival of these early postmitotic granule precursors, and induces granule cell NT-3 expression and neurite outgrowth (Segal et al., *Proc. Nat. Acad. USA* 91:12867-12871 (1994); Segal et al., (1995) supra; Leingärtner et al., *J. Biol. Chem.* 269:828-30 (1994); Lindholm et al., *Eur. J. Neurosci.* 5:1455-64 (1993). At later stages of development, NT-3 promotes neurite fasciculation of granule cells that express increasing amounts of TrkC, perhaps promoting axonal maturation (Segal et al., (1995) supra). The evidence available to date thus indicates that neurotrophins promote the differentiation or survival of granule cells. Similar actions of neurotrophins have been described for neuronal cells throughout the developing nervous system (Snider, *Cell* 77:627-638 (1994)). Less often, neurotrophins have been found to promote the proliferation of neuronal precursors (Kalcheim et al., *Proc. Natl. Acad. Sci. USA* 89:1661-1665 (1992); DiCicco-Bloom et al., *Neuron* 11:1101-1111 (1993)). Medulloblastomas have been found to express NT-3 and its specific receptor TrkC. In a series of 12 patients, it was found that medulloblastomas expressing high levels of trkC have a favorable prognosis (Segal et al., 1994) supra).

Summary of the Invention

The invention is based on the discovery that neurotrophin-3 (NT-3) induces apoptosis in cultured medulloblastoma cells and apoptosis is highly correlated with the level of TrkC gene expression in tumor biopsy samples. NT-3 and its specific receptor TrkC are expressed by the majority of medulloblastomas and TrkC RNA is expressed principally by neoplastic cells within the tumors. Furthermore, the expression of high levels

of TrkC RNA in medulloblastoma has been correlated with a favorable prognosis.

In general, the invention relates to a method for analyzing a medulloblastoma comprising obtaining a medulloblastoma sample and analyzing the sample for apoptosis. This can be used, for example, to evaluate a patient's prognosis or as an aid to determining a treatment modality. The sample can contain living cells, in which case, the sample can be to exposed to NT-3, an NT-3 agonist, or NT-3 modulator before examining the sample for apoptosis.

In order to evaluate the sensitivity of a medulloblastoma to NT-3 (e.g., whether NT-3 induces apoptosis) a medulloblastoma sample can be analyzed for the presence of TrkC. Based on the amount of TrkC present in the sample, the sensitivity of the medulloblastoma to NT-3 is predicted. For example, relatively high levels of TrkC indicate that the medulloblastoma is likely to be sensitive to NT-3. In general, NT-3 sensitivity in a medulloblastoma means that apoptosis is induced by NT-3.

The invention also features a kit useful for assaying a medulloblastoma comprising a supply of NT-3 or a suitable NT-3 agonist, and a supply of reagents and/or apparatus for detecting apoptosis.

Another feature of the invention is a method of screening candidate substances for their potential activity as a treatment for a medulloblastoma. In this method, candidate compounds are added to a screening system that comprises TrkC, and determining whether the candidate substance binds to TrkC. Other methods of screening for such substances are included in the invention. Screening for candidate substances to treat a medulloblastoma can be done using a cell culture (e.g., a granule cell culture system or a primary culture system

derived from a medulloblastoma). A candidate substance that induces apoptosis in a cultured cell is indicated to be of therapeutic value. The candidate substance can be an NT-3 agonist or modulator.

The invention features a method for treating a medulloblastoma comprising administering a therapeutically effective amount of NT-3, an NT-3 agonist, or an NT-3 modulator in a pharmaceutically acceptable carrier to a patient with a medulloblastoma. The method of treatment may involve administering NT-3, an NT-3 agonist, or an NT-3 modulator directly to the central nervous system of the patient. The method of treatment can be supplemented by the additional step of raising the levels of TrkC expression in the medulloblastoma. Various methods of increasing the level of TrkC in a medulloblastoma may be employed, for example, TrkC levels can be raised by administering a nucleic acid encoding a TrkC such as that illustrated by SEQ ID NO:3. A medulloblastoma can also be treated by administering to a patient with a medulloblastoma a nucleic acid comprising SEQ ID NO:1 or a fragment thereof. The nucleic acids used in treatment of a medulloblastoma can be delivered near or directly to the medulloblastoma.

Medicaments can be made for treating disorders involving NT-3, e.g., medulloblastoma. Such medicaments can include an NT-3, an NT-3 agonist, an NT-3 modulator, or a nucleic acid sequence encoding an NT-3 or fragment of an NT-3 encoding sequence.

By "protein" or "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

By "malignancy" is meant any neoplasm or abnormal tissue that grows by cellular proliferation more rapidly

than normal or that continues to grow after growth stimuli cease. Most malignancies show a partial or complete lack of structural organization or functional coordination with surrounding normal tissue. A malignancy according to the invention is generally either locally invasive or metastatic.

"Agonist" refers to an NT-3 analog which binds to TrkC or other receptor with which NT-3 interacts, and mimics NT-3 in that the analog is capable of at least one biological activity associated with NT-3.

"Antagonist" refers to an NT-3 analog which binds to TrkC or other receptor with which NT-3 interacts and inhibits at least one biological function associated with NT-3.

By "modulator" is meant an agent which can elicit the NT-3 apoptotic response by a method other than binding to the NT-3 receptor, for example, by directly phosphorylating TrkC or increasing expression of NT-3.

By "immunological methods" is meant any assay involving antibody-based detection techniques including, without limitation, immunoblotting (e.g., Western blotting), immunoprecipitation, and direct and competitive enzyme linked immunosorbent assay (ELISA), and radioimmunoassay (RIA) techniques.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In

addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figure 1A is a Kaplan-Meir plot of the percentages of children with high or low trkC levels that were free from relapse after diagnosis of medulloblastoma.

Figure 1B is a Kaplan-Meir plot of the percentages of children with high or low trkC levels surviving after diagnosis of medulloblastoma.

Figure 2 is a plot showing the quantitation of nuclear condensation and fragmentation in TrkC-expressing Daoy cells in the presence of NT-3, BDNF, NGF, or no treatment.

Figure 3A depicts the quantitation of nuclear condensation and fragmentation in primary tumor cells expressing low levels of TrkC in the presence of NT-3, BDNF, NGF, or control vehicle.

Figure 3B depicts the quantitation of nuclear condensation and fragmentation in primary tumor cells expressing high levels of TrkC in the presence of NT-3, BDNF, NGF, or control vehicle.

Figure 4 is a plot representing the average proportion of TUNEL-positive nuclei and their correlation with trkC expression in medulloblastoma samples.

Description of the Preferred Embodiments

The invention is related to the discovery that NT-3 promotes apoptosis in medulloblastoma cells. Nearly all medulloblastomas express TrkC, which is the preferred receptor for NT-3. The expression of high levels of TrkC is correlated with a favorable prognosis. It was also

found that medulloblastomas with relatively high levels of TrkC expression appear to undergo more apoptosis.

Northern analysis demonstrated that most medulloblastomas express TrkC and NT-3 (Example 1). By use of *in situ* hybridization, TrkC was shown to be expressed predominantly by tumor cells in medulloblastoma tissue sections (Example 1). Using tissue samples from a series of 35 patients with medulloblastoma, the expression of TrkC RNA was found to be inversely correlated with the presence of metastatic disease at the time of diagnosis, and directly related to a favorable outcome (Examples 2 and 3). Medulloblastomas grown *in vitro* in the presence of NT-3, were found to undergo apoptosis (Example 4). Finally, apoptosis in tumor biopsy sections was found to be highly correlated with TrkC expression (Example 5). Without committing to any particular theory, these results suggest that TrkC receptors alter the growth of medulloblastomas by enhancing programmed cell death, e.g., apoptosis.

Tumor therapy by the induction of programmed cell death with naturally occurring growth factors (e.g., NT-3) may allow the successful treatment of medulloblastoma without the short-term side effects or the long term sequelae of conventional therapies.

Methods for analyzing medulloblastomas

The invention is useful for identifying medulloblastomas likely to be sensitive (e.g., in which apoptosis is induced) to NT-3, NT-3, analogs, NT-3 modulators, TrkC agonists, or other factors activating biochemical pathways involving TrkC or NT-3. The method of detecting sensitive medulloblastomas relies on the observation that high levels of TrkC expression are associated with apoptosis as well as a favorable prognosis in medulloblastoma patients, and that NT-3

induces apoptosis in medulloblastoma cells. Thus, the invention is also useful for predicting the clinical outcome for medulloblastomas subjected to various treatment regimens.

One method of analyzing medulloblastomas as per the invention involves the examination of tumor samples (e.g., biopsies) for the presence of apoptosis. A patient whose medulloblastoma has a greater amount of apoptosis has a better prognosis than a patient whose tumor has little or no apoptosis. Apoptosis may be assayed in a number of ways familiar to those with skill in the art. For example, kits are commercially available for analyzing apoptosis in tissue and cell samples. A specific example is TdT-Mediated dUTP Nick-End Labelling (TUNEL) in which paraffin sections from a medulloblastoma are labeled for nucleosomal DNA degradation by DNA 3'-OH end labelling with fluorescein-conjugated antibodies (ApopTag, Oncor, Gaithersburg, MD; Apoptosis Detection System, Promega, Madison, WI). Other appropriate methods of analyzing apoptosis may be used, such as binding to annexin V (R & D Systems; Minneapolis, MN).

A medulloblastoma may be characterized by analysis of the expression of proteins induced during apoptosis (e.g. Apoptosis-Specific Protein (ASP); Grand et al., *Exp. Cell Res.* 218:439-451 (1995)). For example, expression of apoptotic proteins by medulloblastomas may be analyzed by use of immunocytochemical methods. Antibodies that recognize, for example, ASP are specifically bound to cells or sections from medulloblastomas. The antibodies may be directly or indirectly labeled. For example, a murine antibody that recognizes ASP may be detected using an indirect technique. A sample labeled with the murine antibody is incubated with a second antibody that specifically binds murine antibodies and is covalently linked to a

fluorescent molecule, for example, fluorescein isothiocyanate (FITC), rhodamine, or Cy-3. The labeled antibody complex can be detected using a fluorescent microscope. Such methods are known to those in the art, for example, see Coligan et al., 1994, Current Protocols in Immunology, John Wiley & Sons, Inc.

Detection of apoptosis can also be accomplished by detection transcripts induced by apoptosis, for example, by use of *in situ* hybridization to detect transcripts in cultured cells or sections from a medulloblastoma. Northern blot analysis can also be used to analyze expression. Both *in situ* hybridization and Northern blot analysis are techniques known to those with skill in the art (for example, see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience, New York, 1995), and examples are given below (see Example 1).

Various fragments of apoptosis-induced nucleic acid molecules can be used for detection of expression. These include nucleic acid sequences encoding, most preferably, human apoptosis-induced protein sequences, but also include other apoptosis-induced protein sequences including mouse, rat, and chicken. Preferably, high stringency hybridization conditions are used. Such conditions include hybridization at about 42°C and about 50% formamide, a first wash at about 65°C, in about 2 X SSC, and 1% SDS, followed by a second wash at about 65°C in and about 0.1% SDS and 1 X SSC. Lower stringency conditions for detecting nucleic acid fragments that are short are based on their length, base composition, and empirical observation. For example, hybridization at about 42°C in the absence of formamide, a first wash at about 42°C, in about 6 X SSC, and about 1% SDS, and a second wash at about 50°C, in about 6 X SSC, and about 1% SDS. These conditions are exemplary; other appropriate

conditions can be determined by those skilled in the art.

The invention can also be used to examine medulloblastomas for sensitivity to NT-3. This can be accomplished, for example, by removing a vital sample from a medulloblastoma, culturing cells from that sample, exposing the cells to NT-3, an NT-3 analog, or modulator, and analyzing the culture for apoptosis. Methods for the culture of medulloblastoma cells, in tissue culture, slice culture, or as isolated cells, are described below and are known to those in the art. For example, medulloblastoma cells are dissociated by trituration and grown for one to four days in suspension culture in Dulbecco's Modified Eagle's Medium (DMEM; Gibco/BRL) containing 10% heat inactivated fetal calf serum at 37° and 5% CO_2. NT-3 is then added to the cultures to a final concentration of about 10-100 ng/ml. The cells are then incubated for one to four days and collected by cytocentrifugation for analysis. NT-3, is a peptide that has been cloned and sequenced (for example, GenBank Accession Number M37763; SEQ ID NO:1; SEQ ID NO:2). Thus NT-3 peptides may be synthesized using recombinant or chemical methods known to those with skill in the art.

Another method useful for the analysis of a medulloblastoma is the measurement of expression of a gene or polypeptide induced by NT-3 (e.g., via activation of TrkC). For example, a cultured cell derived from a medulloblastoma is incubated with NT-3 and the ability of NT-3 to activate TrkC is assessed by measuring cFos expression. For example, cFos expression can be assayed by Northern blot, *in situ* hybridization, or immunocytochemical methods as described herein. cFos nucleic acid and polypeptide sequences are described by, for example, GenBank Accession Numbers X06769 and V00727, and Swiss-Prot Accession Number P01100.

The invention includes analysis of medulloblastoma *in situ*. For example, NT-3 or other TrkC ligands are labeled with iodine-123 using known techniques (Wolf et al. *Eur. J. Nucl. Med.* 20:297-301 (1993), injected into individuals, and the posterior fossa imaged with a gamma-camera. A high density image in the posterior fossa indicates a relatively increased likelihood of high TrkC, thus a relatively good prognosis.

The invention may also be used to construct a kit useful for the analysis of medulloblastomas using some or all of the components described above.

Methods of screening for molecules that modulate NT-3 or TrkC

NT-3 agonists and modulators are useful for the treatment of medulloblastoma and other NT-3-sensitive malignancies, as are TrkC agonists and modulators. The invention is useful for the identification of such molecules.

The following assays are designed to identify compounds that are effective ligands (preferably agonists) or modulators of NT-3 or TrkC. Preferably, such compounds increase NT-3 expression or activity. Increasing TrkC expression or activity is also useful for the invention. Such modulators may act by, but are not limited to, binding to a TrkC molecule, binding to proteins that bind to a TrkC molecule, compounds that enhance the interaction between a NT-3 molecule and a TrkC molecule, compounds that modulate the activity of an NT-3 or TrkC molecule, or modulate the expression of NT-3 or TrkC.

Assays can also be used to identify molecules that bind to NT-3 or TrkC gene regulatory sequences (e.g., promoter sequences), thus modulating gene expression.

See e.g., Platt, *J. Biol. Chem.* 269:28558-28562 (1994), incorporated herein in its entirety.

The compounds which can be screened by the methods described herein include, but are not limited to, peptides and other organic compounds (e.g., peptidomimetics) that bind to an NT-3 or TrkC protein, or promote their activity in any way. Such peptide compounds may include, but are not limited to, for example, soluble peptides, including but not limited to members of random peptide libraries; (see, e.g., Lam et al, 1991. *Nature* 354:82-84; Houghten et al., 1991. *Nature* 354:84-86), and combinatorial chemistry-derived molecular libraries made of D-and/or L- amino acids, phoshopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries; see, e.g., Songyang et al., 1993. *Cell* 72:767-778), and small organic or inorganic molecules.

Organic molecules are screened that affect expression of a NT-3 or TrkC gene or some other gene involved in the regulatory pathway for NT-3 or TrkC (e.g., by interacting with the regulatory region or transcription factors of a gene). Compounds are also screened that affect the activity of such proteins, (e.g., by enhancing NT-3 activity) or the activity of a molecule involved in the regulation of an NT-3 or TrkC.

In one approach, computer modelling or searching technologies are used to identify compounds, or identify modifications of compounds that modulate the expression or activity of a NT-3 or TrkC protein. For example, compounds likely to interact with the active site of a protein (e.g., with a site where NT-3 and interact TrkC) are identified. The active site of a molecule can be identified using methods known in the art including, for example, analysis of the amino acid sequence of a molecule, from a study of complexes with the relevant

compound, or composition with its native ligand. Chemical or X-ray crystallographic methods can be used to identify an active site by the location of the bound ligand.

The three-dimensional structure of an active site may be determined using known methods, including X-ray crystallography which may be used to determine a complete molecular structure. Solid or liquid phase NMR can be used to determine certain intra-molecular distances. Other methods of structural analysis can be used to determine partial or complete geometrical structures. Geometric structure may be determined with a TrkC bound to a natural (e.g., NT-3) or artificial ligand, which may provide a more accurate active site structure determination.

Computer-based numerical modelling can be used to complete an incomplete or insufficiently accurate structure. Modelling methods that may be used are, for example, parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups are necessary, and can be selected from force fields known in physical chemistry. Information on incomplete or less accurate structures determined as above can be incorporated as constraints on the structures computed by these modeling methods.

Having determined the structure of the active site or sites of a TrkC protein, either experimentally, by modelling, or by a combination of methods, candidate modulatory compounds can be identified by searching databases containing compounds along with information on their molecular structure. The compounds identified in

such a search are those that have structures that match the active site structure, fit into the active site, or interact with groups defining the active site. These compounds are likely candidates for NT-3/TrkC ligands or modulating compounds.

These methods may also be used to identify an improved ligand or modulating compound from an already known modulating compound or ligand. The structure of the known compound is modified and effects are predicted or determined using computer modelling and experimental methods as described herein. The altered structure may be compared to the active site structure of an NT-3 or TrkC molecule to predict or determine how a particular modification to the ligand or modulating compound will affect its interaction with that protein. Systematic variations in composition, such as by varying side groups, can be evaluated to obtain modified modulating compounds or ligands of preferred specificity or activity.

Other experimental and computer modeling methods useful to identify a ligand or modulating compound based on identification of the active sites of an NT-3 or TrkC molecule and related transduction and transcription factors will be apparent to those of skill in the art.

Examples of molecular modelling systems are the QUANTA programs, e.g., CHARMm, MCSS/HOOK, and X-LIGAND, (Molecular Simulations, Inc., San Diego, CA). QUANTA analyzes the construction, graphic modelling, and analysis of molecular structure. CHARMm analyzes energy minimization and molecular dynamics functions. MCSS/HOOK characterize the ability of an active site to bind ligand using energetics calculated via CHARMm. X-LIGAND fits ligand molecules to electron density of protein-ligand complexes. It also allows interactive construction,

modification, visualization, and analysis of the behavior of molecules with each other.

Articles reviewing computer modelling of compounds interacting with specific proteins can provide additional guidance. For example, see, Rotivinen et al, 1988, *Acta Pharmaceutical Fennica* 97:159-166; Ripka, *New Scientist* 54-57 (June 16, 1988); McKinaly and Rossmann, 1989, *Ann. Rev. Pharmacol. Toxicol.* 29:111-122; Perry and Davies. *OSAR: Quantitative Structure-Activity Relationships in Drug Design* pp. 189-193 (Alan R. Liss, Inc., 1989); Lewis and Dean, 1989, *Proc. R. Soc. Lond.* 236:125-140, 141-162; and, regarding a model receptor for nucleic acid components, Askew et al., *Am. J. Chem. Soc.* 111:1082-1090. Computer programs designed to screen and depict chemicals are available from companies such as MSI (*supra*), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Gainesville, FL). These applications are largely designed for drugs specific to particular proteins, they may be adapted to the design of drugs specific to identified regions of DNA or RNA.

In addition to designing and generating compounds that alter binding, as described above, libraries of known compounds, including natural products, synthetic chemicals, and biologically active materials including peptides, can be screened for compounds that are inhibitors or activators. Candidate NT-3 ligands or modulators also include peptide as well as non-peptide molecules (e.g., compounds found in a cell extract, mammalian serum, or growth medium in which mammalian cells have been cultured).

The effects of such molecules can be tested by a number of methods. According to one approach, a candidate modulator of NT-3 gene expression is added at varying concentrations to the culture medium of cells capable of expressing NT-3 mRNA. NT-3 expression is then

measured, for example, by standard Northern blot analysis (Ausubel et al., *supra*) using a labeled NT-3 cDNA (or cDNA fragment) as a hybridization probe. The level of NT-3 expression in the presence of the candidate modulator molecule is compared to the level measured for the same cells in the same culture conditions but in the absence of the candidate molecule.

If desired, the effect of candidate modulators on expression can, in the alternative, be measured at the level of NT-3 protein production using the same general approach as is described above and standard immunological detection techniques, such as immunoblotting or immunoprecipitation with an NT-3-specific antibody (for example, using the NT-3 antibodies and methods described herein) to detect relative amounts of NT-3. NT-3-specific antibodies have been described (Barres et al., *Nature* 367:371 (1994)). An increase in detectable NT-3 indicates that the candidate modulator may be useful for increasing NT-3 levels.

Candidate modulators or ligands can be purified (or substantially purified) molecules or can be one component of a mixture of compounds (e.g., an extract or supernatant obtained from cells; Ausubel et al., *supra*). In a mixed compound assay, NT-3 expression is tested against progressively smaller subsets of the candidate compound pool (e.g., produced by standard purification techniques, e.g., HPLC or FPLC) until a single compound or minimal compound mixture is demonstrated to modulate NT-3 expression. Additional assay methods are provided below.

A molecule that promotes an increase in NT-3 expression or activity is considered useful in the invention; such a molecule can be used, for example, as a therapeutic to increase cellular levels of an NT-3 polypeptide or to increase NT-3 binding activity and

thereby exploit the effect of NT-3 on medulloblastoma cells.

NT-3 appears to activate apoptosis in medulloblastoma via its interaction (e.g., binding and subsequent phosphorylation) with TrkC. Thus, molecules that increase TrkC activity, for example, through tyrosine phosphorylation are useful. These molecules may be identified using methods similar to those described above. For example, using methods known in the art, agents that act as tyrosine kinases can be assayed for their ability to induce apoptosis in medulloblastoma cells. As described above for NT-3 ligands and modulators, such candidate molecules may be identified and then tested for efficacy in a biochemical system. For example, an increase in TrkC gene expression can be assayed by adding a candidate TrkC ligand modulator in varying concentrations to the culture medium of cerebellar granule cells. After incubation in the presence of a candidate molecule, TrkC expression is measured, for example, by Northern blot analysis (Ausubel et al., *supra*) using a labeled TrkC cDNA (or cDNA fragment) as a hybridization probe. A TrkC cDNA probe can be based on any TrkC nucleic acid sequence, but preferably human, for example, those depicted in GenBank accession numbers HSU05012 (SEQ ID NO:3), S76475, or S76476. The level of TrkC expression in the presence of the candidate modulator molecule is compared to the level measured for the same cells grown under the same culture conditions but in the absence of the candidate molecule. An increase in the amount of TrkC mRNA in those cells incubated with the candidate modulator indicates that the candidate modulator is a candidate compound for use in the treatment of a medulloblastoma.

If desired, the effect of candidate modulators on TrkC expression can be measured at the level of TrkC

protein production using the same general approach as is described above, and standard immunological detection techniques, such as immunoblotting or immunoprecipitation with a TrkC-specific antibody (for example, using the methods described herein) to detect relative amounts of TrkC. TrkC-specific antibodies have been described (Amgen; David Kaplan, Montreal Neurologic Institute). An increase in detectable TrkC indicates that the candidate modulator may be useful for increasing TrkC levels in a patient with a medulloblastoma. Additional assay methods are provided below. Without committing to any particular theory, these molecules are useful for increasing the amount of TrkC in medulloblastomas having low amounts of TrkC, by increasing the affinity of TrkC for NT-3, increasing the activity of existing levels of TrkC, or by obviating the need for NT-3 in TrkC activation.

Compounds identified by methods described above may be useful, for example, for elaborating the biological function of NT-3 or TrkC gene products, and in treatment of disorders involving NT-3 and TrkC (e.g., a medulloblastoma). Additional assays for testing the effectiveness of compounds such as those described herein are described below.

In vitro screening assays for compounds that bind to NT-3 or TrkC proteins and genes

In vitro systems may be used to identify compounds that can interact (e.g., bind) with NT-3 or TrkC proteins or genes encoding those proteins. Such compounds may be useful, for example, for modulating the activity of these entities, elaborating their biochemistry, or treating disorders involving these entities (e.g., a medulloblastoma). These compounds may be used in screens for other compounds that enhance or disrupt normal

function, or may themselves enhance or disrupt normal function.

Assays to identify compounds that bind NT-3 or TrkC proteins involve preparation of a reaction mixture of the protein and the test compound under conditions sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected.

Screening assays may be performed using a number of methods. For example, an NT-3 or TrkC protein, peptide, or fusion protein can be immobilized onto a solid phase, reacted with the test compound, and complexes detected by direct or indirect labeling of the test compound. Alternatively, the test compound can be immobilized, reacted with an NT-3 or TrkC molecule, and the complexes detected. Microtiter plates may be used as the solid phase and the immobilized component anchored by covalent or noncovalent interactions. Non-covalent attachment may be achieved by coating the solid phase with a solution containing the molecule and drying. Alternatively, an antibody, for example, one specific for NT-3 or TrkC is used to anchor the molecule to the solid surface. Such surfaces may be prepared in advance of use, and stored.

In the assay, the non-immobilized component is added to the coated surface containing the immobilized component under conditions sufficient to permit interaction between the two components. The unreacted components are then removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid phase. The detection of the complexes may be accomplished by a number of methods known to those in the art. For example, the nonimmobilized component of the assay may be prelabeled with a radioactive or enzymatic entity and detected using

appropriate means. If the non-immobilized entity was not prelabeled, an indirect method is used. For example, if the non-immobilized entity is a TrkC polypeptide, an antibody that recognizes the TrkC polypeptide is used to detect the bound molecule, and a secondary, labeled antibody used to detect the entire complex.

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected (e.g., using an immobilized antibody specific for an NT-3 or TrkC polypeptide.

Cell-based assays may be used to identify compounds that interact with TrkC or NT-3 polypeptides and so may be useful for the invention. Cell lines that naturally express such proteins or have been genetically engineered to express such proteins (e.g., by transfection or transduction of NT-3 or TrkC DNA) can be used. For example, test compounds may be administered to cell cultures and the amount of transcription from an NT-3 gene analyzed, e.g., by Northern analysis. An increase in the amount of RNA transcribed from such a gene compared to control cultures that did not contain the test compound indicates that the test compound is an inducer of NT-3 expression.

Assays for compounds that modulate the effects of NT-3 or TrkC in vivo

Compounds identified as above, or other candidate compounds that enhance NT-3 and/or TrkC activity or expression *in vitro* may be useful for treating a medulloblastoma. These compounds may be tested *in vivo*, for example, in animal models of medulloblastoma. For example, nude mice can be injected with medulloblastoma cells (xenograft; Friedman et al., *Am. J. Path.* 130:472-84 (1988), Trojanowski et al., *Molec. Chem. Neuropath.*

21:219-239 (1994)). Test compounds predicted to enhance NT-3 or TrkC expression or activity are administered to an animal with a medulloblastoma and assayed for apoptosis in a medulloblastoma, decreased tumor size, or any other sign of slowed or arrested tumor development, as described herein. Such assays may be indirect or inferential, for example, improved health or survival of the animal. Assays may also be direct, for example, measuring an increase in NT-3 expression by Northern analysis on tissue removed from an animal treated with a test compound. An increase in the amount of NT-3 mRNA present in the sample from treated animals compared to untreated control indicates that the test compound is enhancing NT-3 expression *in vivo*.

Methods for the treatment of medulloblastoma

The invention also features the treatment of medulloblastomas with NT-3, NT-3 analogs, or NT-3 modulators. The treatment may be delivered to the patient parenterally or orally in an appropriate pharmaceutical composition.

Treatment of a medulloblastoma may also be effected by direct delivery of NT-3 or NT-3 analogs to the central nervous system, preferentially to the brain, and in a more preferred embodiment, near to or directly at the site of the medulloblastoma. Accordingly, NT-3 or analogs may be formulated into a pharmaceutical composition by admixture with pharmaceutically acceptable nontoxic excipients or carriers. Such a composition can be prepared for use in parenteral administration or for oral administration. Formulations for parenteral administration can contain as common excipients sterile water or saline solution, polyalkylene glycols, vegetable oils, and the like. Biocompatible, biodegradable

polymers may be useful excipients to control the release of the active compound(s).

A variety of drug delivery systems can be used in practicing the present invention. Potentially useful parenteral delivery systems for the active compounds include slow-dissolving polymer particles, implantable infusion systems, and liposomes.

A preferred parenteral route of administration of NT-3, NT-3 agonists, or NT-3 modulators is directly into or near the tumor (e.g., into the fourth ventricle). In some cases, e.g., when the tumor is metastatic, administration into the CNS may be desirable, (e.g., intrathecal or intracerebral ventricular). For example, an Omaya reservoir-shunt with in-line filter can be surgically placed into the cisternal space. NT-3 in an appropriate excipient (e.g. phosphate-buffered saline) is instilled into the shunt by injection on a prescribed basis.

The concentrations of the active ingredients in the composition will depend on factors including the total number of active ingredients present, the dosage of the active ingredients to be delivered, the chemical characteristics of the active ingredients, and the route of administration.

Preferably the dose range for NT-3 is from 25 to 500 micrograms per day.

The effectiveness of a particular composition can be ascertained in a particular patient by a number of methods. For example, the size of the medulloblastoma can be evaluated using imaging methods (e.g., MRI), the assessment of physical symptoms of the patient, or biopsy of the tumor. In the case of effective treatment, any of the following may occur. Tumor growth will slow, stop, or can decrease. Increasing intracranial pressure and associated symptoms (e.g., headache, vomiting,

papilloedema, ataxia, or cranial nerve deficits) may slow, stop, and may improve. Biopsy may reveal increased apoptosis of responsive tumor tissue.

Another mode of administering NT-3 to a patient with medulloblastoma comprises introduction of a functional NT-3 nucleic acid into cells (e.g., SEQ ID NO:1). For example, a functional NT-3 gene can be introduced into cells at the site of a tumor.

Retroviral vectors, adenoviral vectors, adeno-associated viral vectors, or other viral vectors with the appropriate tropism for NT-3 responsive cells (for example, granule cells) can be used as a gene transfer delivery system for a therapeutic NT-3 gene construct. Numerous vectors useful for this purpose are generally known (Miller, *Human Gene Therapy* 15-14, (1990); Friedman, *Science* 244:1275-1281, (1989); Eglitis and Anderson, *BioTechniques* 6:608-614, (1988); Tolstoshev and Anderson, *Current Opinion in Biotechnology* 1:55-61, (1990); Sharp, *The Lancet* 337:1277-1278, (1991); Cornetta et al., *Nucleic Acid Research and Molecular Biology* 36:311-322, (1987); Anderson, *Science* 226:401-409, (1984); Moen, *Blood Cells* 17:407-416, (1991); and Miller and Rosman, *BioTechniques* 7:980-990, (1989); Le Gal La Salle et al., *Science* 259:988-990, (1993); and Johnson, *Chest* 107:77S-83S, (1995)). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., *N. Engl. J. Med* 323:370, (1990); Anderson et al., U.S. Pat. No. 5,399,346).

Non-viral approaches can also be employed for the introduction of therapeutic DNA into malignant cells. For example, an NT-3 gene can be introduced into a medulloblastoma cell by the techniques of lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413, (1987); Ono et al., *Neuroscience Lett.* 117:259, (1990);

Brigham et al., *Am. J. Med. Sci.* 298:278, (1989); Staubinger and Papahadjopoulos, *Meth. Enzymol* 101:512, (1983)); polylysine conjugation methods (Wu and Wu, *J. Biol. Chem.* 263:14621, (1988); Wu et al., *J. Biol. Chem.* 264:16985, (1989)); or, by microinjection under surgical conditions (Wolff et al., *Science* 247:1465, (1990)).

For any of the above approaches, the therapeutic NT-3 DNA construct is preferably applied to the target area (e.g., a medulloblastoma), but can also be applied in the vicinity of the target area, for example, the fourth ventricle.

For gene therapy, NT-3 cDNA expression is directed from any suitable promoter (e.g., the human cytomegalovirus, simian virus 40, or metallothionein promoters), and its production is regulated by any desired mammalian regulatory element. For example, if desired, enhancers that direct preferential gene expression in granule cells can be used to direct NT-3 expression.

Alternatively, if an NT-3 genomic clone is utilized as a therapeutic construct, NT-3 expression is regulated by its cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, e.g., any of the promoters or regulatory elements described herein.

NT-3 gene therapy is also accomplished by direct administration of an NT-3 mRNA to a target area (e.g., a medulloblastoma). This mRNA can be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using an NT-3 cDNA under the control of a high efficiency promoter (e.g., the T7 promoter). Administration of NT-3 mRNA to malignant cells is carried out by any of the methods for direct nucleic acid administration described above.

Other gene therapy methods such as the use of mammalian artificial chromosomes (MACs; Harrington et al., *Nature Genetics*, 15:345-355 (1997)) may be useful for raising the levels of NT-3 in NT-3-sensitive tumors.

The production of NT-3 polypeptide by any gene therapeutic approach described above results in a cellular level of NT-3 that is at least equivalent to the normal, cellular level of NT-3 in an unaffected individual.

The effects of NT-3 therapy (e.g., by administration of NT-3 or NT-3 gene therapy) can be enhanced by increased expression or activity of TrkC. This may be accomplished by the delivery of TrkC polypeptides or fragments thereof, TrkC analogs, modulators, or gene therapy in combination with NT-3 treatments, as described above. A preferred method for delivering TrkC to cells is lipofection. Methods for identifying appropriate TrkC molecules for use in the invention are analogous to those described above for NT-3. Gene therapy with trkC (e.g., SEQ ID NO:3) and nucleic fragments thereof is accomplished as described above for NT-3.

For the treatment of a medulloblastoma, treatment by any NT-3-mediated method as described herein can be combined with more traditional cancer therapies such as surgery, radiation, or chemotherapy. The efficacy of any of the above treatments is monitored as described above.

The nucleic acid sequences used for the invention (e.g., for detection by Northern analysis, *in situ* hybridization, and gene therapy) may be derived from any source including but not limited to mammals such as humans, mice, rats, or other metazoans such as chickens and Drosophila, and can be fragments or entire sequences. Both NT-3 and TrkC are both known and are identified by GenBank Accession numbers, for example M37763 and S55222

(human NT-3 genes), U05012 (SEQ ID NO:3; SEQ ID NO:4) (human receptor tyrosine kinase TrkC mRNA), and S76476 (human brain trkC mRNA, alternatively spliced).

Examples

Example 1. Expression of neurotrophins and Trks in medulloblastomas

Northern analysis of whole cell RNA was used to examine the expression of neurotrophins and Trks in medulloblastomas. Medulloblastoma samples for this study came from all patients treated for medulloblastoma at Boston Children's Hospital and the Dana-Farber Cancer Institute from June 1993 to June 1996. Additional samples were obtained from patients treated at New York University Medical School and at the New England Medical Center. Samples were snap frozen in liquid nitrogen and stored at -80°C until further analysis. All samples with a sufficient amount of adequately preserved tumor tissue were included in the study. In all cases, the diagnosis of medulloblastoma was confirmed by pathologic analysis of biopsy samples. At the time of diagnosis, the patients ranged in age from 7 - 324 months (mean = 107 months). There were 11 females and 24 males. All patients were treated with craniospinal irradiation with a 5300 - 7200 cGy tumor dose and 2400 - 3600 cGy craniospinal dose. All but 3 patients were treated with chemotherapy consisting of cisplatin or carboplatin, and combinations of vincristine, etoposide, cyclophosphamide or CCNU. Two patients received high dose chemotherapy, one as primary therapy and the other at relapse, including methotrexate and thiotepa followed by autologous bone marrow transplantation.

Total cellular RNA was isolated on CsCl gradients after homogenization of fresh frozen tumor samples in guanidine isothiocyanate. Northern analysis and

quantification of mRNA expression was done as described previously (Segal et al., (1994) supra). Trk probes were generated from cDNAs encoding sequences from the extracellular domains of human TrkB and TrkC (courtesy of David Shelton, Genentech, San Francisco, CA). All other probes have been described previously (Segal et al., (1994) supra). Northern analysis of the biopsy samples demonstrated that all of the tumors expressed a 14.0 kb trkC transcript as well as a 5.5 kb alternative splice variant, both of which encode the full length $p145^{trkC}$ receptor (Tsoulfas et al., *Neuron* 10:97590 (1993); Valenzuela et al., *Neuron* 10:963-974 (1993); Shelton et al., *J. Neurosci.* 15:477-491 (1995)). All but eight of the tumors expressed NT-3, the preferential ligand for TrkC (Tsoulfas et al., supra).

In contrast to trkC expression, the predominant trkB alternative splice variant, expressed in 26 of the 35 tumors, was a 7.0 kb transcript that encodes a truncated receptor lacking a tyrosine kinase domain (Shelton et al., supra; Middlemas et al., *Mol. Cell. Biol.* 11:143-153 (1991)). The 9.0 kb transcript that encodes the full-length $p145^{trkB}$ receptor protein was found in 18 of the 35 tumor samples (Middlemas et al., supra). Only 12 of the tumors were found to express BDNF. A total of 11 tumors had measurable levels of p75, the low affinity nerve growth factor receptor. No tumor samples were found to express trkA.

The cellular expression of the neurotrophin receptors was assessed by *in situ* hybridization. Paraffin-embedded tissue sections (10 μm) were hybridized with ^{35}S-labeled anti-sense and sense human Trk riboprobes prepared according to the protocol of Ausubel et al. (Current Protocols in Molecular Biology, New York: Wiley-Interscience, 1995, Unit 14.3.) using the same cDNA sequences as were used for Northern analysis. Tissue

sections were hybridized at 42°C for 18 - 20 hrs. The slides were dipped in NTB emulsion (Kodak, Rochester, NY), exposed at 4°C for 7 days, developed, and counterstained with hematoxylin. They were viewed with both bright and darkfield microscopy.

Biopsy specimens from the twelve patients described in Segal et al. (1994; supra) were analyzed as a representative sample. *In situ* hybridization performed on medulloblastoma cells demonstrated that trkC was expressed predominantly by neoplastic cells within the biopsy specimens. Within each biopsy sample, the expression of trkC was consistent from cell to cell. There were significantly fewer silver grains in the emulsion over neoplastic cells from tumors expressing low levels of trkC, as determined by Northern analysis (see below), than in tumors expressing high levels of trkC (Mann-Whitney U test, $P = 0.002$).

Example 2. Biologic correlates of trkC expression

The level of expression of the 14 kb splice variant of trkC detected by Northern analysis was quantified on a Phosphoimager (Molecular Dynamics, Sunnyvale, CA). An index of expression was calculated for each of the samples as described previously, except that a 28S rRNA oligonucleotide probe was used to control for unequal loading of the samples (Segal et al., (1994) supra; Houge et al., *Mol. Cell. Biol.* 15: 2051-2062 (1995)). The distribution of the indices was highly skewed, and ranged from 0.1 to 45.7 with a median value of 0.80 (mean = 3.9). Thus, the level of expression was highly variable, with a greater than 450-fold difference between the highest and lowest values.

Table 1. Relationship of trkC expression with evidence for metastatic disease at the time of diagnosis.

No

	Positive MRI	Positive CSF cytology	metastatic disease
Low trkC expression	5	4	9
High trkC expression	2	0	15

The sample was dichotomized into groups expressing high or low levels of trkC by dividing at the median. The mean age of patients with low trkC expression was 88 ± 56 months (mean ± S.D.) while the mean age of those with high expression was 127 ± 109 months. This trend was not significant (t test, P = 0.2). There was no relationship between trkC expression and the degree of surgical resection (Fisher's exact test, P = 0.72). The expression of trkC was significantly and inversely associated with metastatic disease at time of diagnosis (Fisher's exact test, P = 0.03).

Patients with tumors expressing high levels of trkC rarely presented with neoplastic cells in the cerebral-spinal fluid or with overt metastases as determined by MRI scanning (see Table 1). Evidence for metastatic disease at the time of diagnosis was found in half of the patients with low trkC expression.

Example 3. High trkC expression predicts a more favorable clinical outcome

Preliminary work in a small sample of patients demonstrated that children with tumors expressing high levels of trkC had fewer relapses and a more favorable overall survival than those with tumors expressing low levels of the receptors (Segal et al., (1994) supra). This was further investigated in a larger patient cohort.

In this larger series, the patients were divided into groups with high and low expression of trkC by dichotomizing at the median index value and calculating survival analysis according to the method of Kaplan and Meier (Kaplan et al., *J. Am. Stat. Assoc.* 53:457-481 (1958)). Kaplan-Meier plots for survival analysis were calculated with the SAS statistical analysis program (SAS Institute, Inc., Cary, NC). The cell counting data was evaluated by ANOVA (StatView; Abacus Concepts, Inc., Berkeley, CA) and a least squares model was used to calculate correlation coefficients (Snedecor et al., Statistical Methods 1980 Iowa State University Press, Ames). Two-tailed tests of significance were used. As shown in Figure 1, both progression-free survival (Fig. 1A) and overall survival (Fig. 1B) were significantly better in children with medulloblastomas that expressed high levels of trkC than children with low levels of tumor receptor expression. The median survival of patients with tumors expressing high levels of trkC was 92 months compared with 39 months for patients with low trkC expression. Evidence for metastatic disease at the time of diagnosis also predicted a shorter progression-free survival ($P = 0.009$) and a worse overall survival ($P = 0.01$). Age, gender, treatment center, and degree of surgical resection were not significantly associated with either progression-free or overall survival. Furthermore, the expression of neither the 7.0 or 9.0 kb alternative splice variants of trkB, p75, BDNF, or NT-3 predicted progression-free or overall survival.

Example 4. <u>Induction of medulloblastoma apoptosis by NT-3 *in vitro*</u>

While the expression of trkC may serve as a marker for the cells from which medulloblastomas arise, perhaps indicating that favorable tumors are derived from more

mature granule cells, the observation that both NT-3 and TrkC are expressed by medulloblastomas implies autonomous TrkC activation that may alter tumor growth. To address whether TrkC activation may affect the growth of the neoplasms, the medulloblastoma cell line Daoy, that normally expresses very low levels of the 5.5 kb trkC alternative splice variant (Segal et al., (1994) supra), was transfected with an expression plasmid encoding $p145^{trkC}$.

The medulloblastoma cell line Daoy was obtained from the American Type Culture Collection (ATCC), and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) heat-inactivated fetal calf serum at 37°C in 5% CO_2. Daoy clones were maintained under the same conditions after stable transfection (Lipofectin; Gibco BRL, Gaithersburg, MD) with a TrkC expression plasmid (Tsoulfas et al., supra; courtesy of Luis Parada, Univ. of Texas). Success of transfection was determined by Western blotting to identify TrkC. Fos induction was used as determined by immunocytochemistry (Segal et al., *Neuron* 9:1041-52 (1992)) was used as evidence of TrkC-induced signalling. To test for neurotrophin response, transfected cells grown on poly-l-lysine coated coverslips were placed in serum-free DMEM for 12-18 hours with neurotrophins added (50 ng/ml; courtesy of Andy Welcher, Amgen, Thousand Oaks, CA). The cells were fixed in 4% paraformaldehyde, stained with Hoechst 33342 and viewed (five high powered fields for each of four replicated coverslips per condition) with a fluorescence microscope (100X objective) with the observer blind to the experimental conditions. Clones of stable transfectants were isolated and tested for the presence of increased TrkC protein expression by immunoprecipitation and for functional receptor by testing for NT-3-induced Fos expression using

immunocytochemistry (Segal et al., (1992) supra). When grown under serum-free conditions, Daoy subclones expressing high levels of TrkC were found to undergo extensive programmed cell death when grown in the presence of 50 ng/ml NT-3 (Fig. 2). This result was repeated in three separate experiments with two different Daoy subclones.

TUNEL labeling was used to confirm that cell death was due to apoptosis. TUNEL labeling was performed by labeling paraffin sections of medulloblastomas for nucleosomal DNA degradation by DNA 3'-OH end labeling using ApopTag (Oncor; Gaithersburg, MD) with fluorescein-conjugated antibodies according to the manufacturers' specifications. The slides were counterstained with propidium iodide and nuclei were counted through a fluorescence microscope under high power (100X objective).

NT-3 induction of programmed cell death was blocked by the addition of the Trk tyrosine kinase inhibitor K252a (100 nM; Calbiochem). In these experiments, two TrkC expressing Daoy subclones did not have significantly increased apoptosis over vehicle control when grown in the presence of NT-3 and K-252a ($P>0.4$ for both, ANOVA), whereas both had significantly increased cell death when grown in the presence of NT-3 alone ($P<0.001$, ANOVA). Neither BDNF (50 ng/ml) nor NGF (50 ng/ml) induced apoptosis in these cell lines (Fig. 2).

To identify whether a similar effect could be observed in primary tumors, NT-3 was tested for its ability to induce apoptosis in primary cultures of medulloblastoma. Tumor biopsy samples were minced, triturated, and placed in serum-free DMEM with neurotrophins (50 ng/ml) or vehicle control. The cells were grown for 12 - 18 hours, fixed in 4%

paraformaldehyde, and dried onto glass microscopy slides. The cells were stained with Hoechst 33342 and viewed with a fluorescence microscope using the strategy noted above.

Cells from a patient with low trkC expression and another with high expression were grown in the presence of NT-3, BDNF, or NGF (each at 50 ng/ml). The cells were grown in suspension, fixed, dried onto glass slides, and stained with Hoechst 33342 for microscopic analysis as described above. Similar to the Daoy subclones, the primary cultures underwent extensive cell death when treated with NT-3 but not with BDNF or NGF (Figs. 3A and 3B).

Example 5. <u>Apoptosis is highly correlated with trkC expression in tissue sections of medulloblastoma</u>

Since expression of both NT-3 and TrkC in medulloblastomas may promote programmed cell death *in vivo,* we examined apoptosis in the 12 tumor samples described in Segal et al., (1994) <u>supra</u>. Hematoxylin-stained biopsy sections were examined to define regions with dense infiltration of tumor cells. Comparable fields in adjacent serial sections were examined for apoptosis and proliferation. Tissue sections from each of these tumors were examined for nucleosomal degradation by TUNEL labeling and for pyknosis following propidium iodide staining. TUNEL labeling was performed as described in Example 4. To verify the TUNEL data, the proportion of pyknotic nuclei in rhodamine stained sections was counted using a 40X objective. For each of these observations, nuclei were counted in ten high-power fields per tumor biopsy sample with the observer blind to the patient's disease status. The proportion of proliferating cells was determined in tissue sections

from the same patients stained with the Ki67 antibody (Dako; Carpinteria, CA).

The proportion of TUNEL-positive nuclei was determined in ten high powered fields (100X objective) per tumor sample, and the proportion of pyknotic nuclei in ten different high powered fields (40X objective). The mean proportion of apoptotic nuclei for each specimen was then compared with trkC expression as determined by Northern analysis, using a least squares model. It was found that apoptosis correlated with trkC expression both by the TUNEL method ($r = 0.80$, $P = 0.003$); see Figure 4) and by nuclear pyknosis ($r = 0.77$, $P = 0.02$). In contrast, expression of neither the 9 kb trkB alternate splice variant ($r = 0.21$, $P = 0.8$) nor the 7.0 kb splice variant ($r = 0.16$; $P = 0.8$) was correlated with apoptosis measured by either method (TUNEL results shown). Proliferation as measured by the portion of Ki67 antibody positive cells, was not significantly correlated with trkC expression ($r = 0.21$, $P = 0.51$).

Other uses of the invention

The invention is also useful for the treatment of other types of neural tumors in which apoptosis is induced by NT-3 or other related methods described above. This includes tumors in which NT-3 interacts with other ligands besides, or in addition to TrkC (e.g., p75 low-affinity nerve growth factor).

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A method for analyzing a medulloblastoma comprising:

a. obtaining a medulloblastoma sample; and

b. analyzing said sample for apoptosis.

2. The method of claim 1 in which analyzing said sample comprises living cells and said method further comprises exposing said sample to NT-3, an NT-3 agonist, or NT-3 modulator before examining said sample for apoptosis.

3. A method for detecting an NT-3-sensitive medulloblastoma comprising:

a. obtaining a medulloblastoma sample;

b. analyzing said sample for the presence of TrkC; and

c. based on the amount of TrkC present in said sample, predicting the sensitivity of said medulloblastoma to NT-3.

4. A kit useful for assaying a medulloblastoma comprising:

a. a supply of NT-3 or a suitable NT-3 agonist;

b. a supply of reagents and/or apparatus for detecting apoptosis.

5. A method of screening candidate substances to determine potential activity to treat a medulloblastoma comprising adding candidate compounds to a screening system that comprises TrkC, and determining whether the candidate substance binds to TrkC.

6. The method of claim 5 in which the screening system is a cell culture and candidate substance induction of apoptosis is indicative of therapeutic value.

7. The method of claim 5 in which the candidate substance is an NT-3 agonist.

8. Use of NT-3, an NT-3 agonist, or an NT-3 modulator in the manufacture of a medicament for treating a medulloblastoma.

9. The use of claim 8 in the manufacture of a medicament to be administered directly to the central nervous system of a patient.

10. The use of claim 8, in the manufacture of a medicament to raise levels of TrkC expression in said medulloblastoma.

11. The use of claim 10, in the manufacture of a medicament comprising a nucleic acid comprising SEQ ID NO:3 or a fragment thereof.

12. Use of a nucleic acid comprising SEQ ID NO:1 or a fragment thereof in the manufacture of a medicament for treating medulloblastoma.

13. The use of claim 11 or claim 12, in the manufacture of a medicament targeted to said medulloblastoma.

14 A medicament comprising an NT-3, an NT-2 antogonist, or an NT-3 modulator.

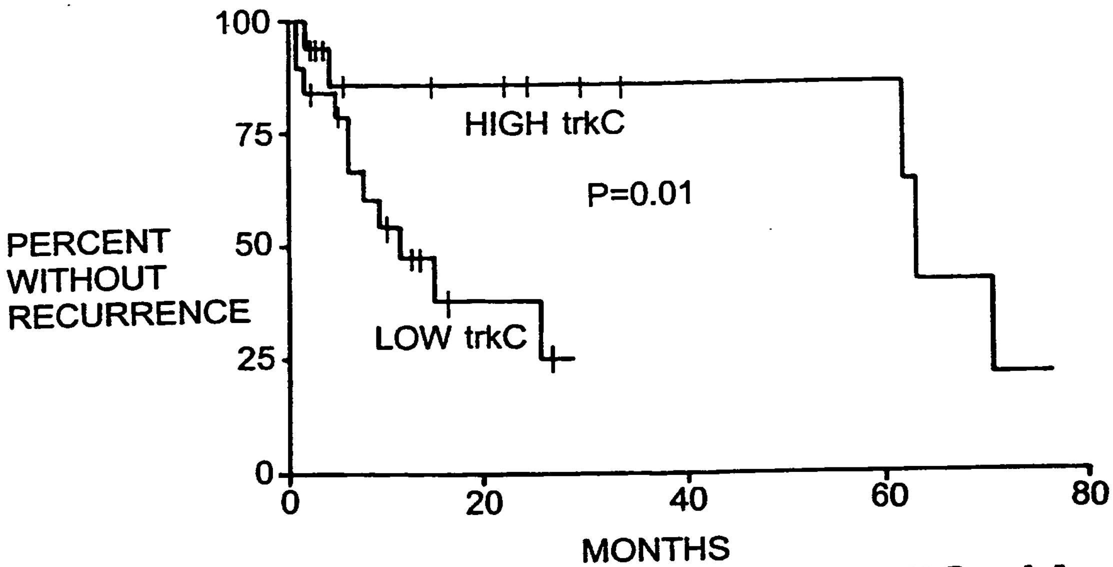

100
75
50
25
0
PERCENT
WITHOUT
RECURRENCE
HIGH trkC
P=0.01
LOW trkC
0
20
40
60
80
MONTHS

FIG. 1A

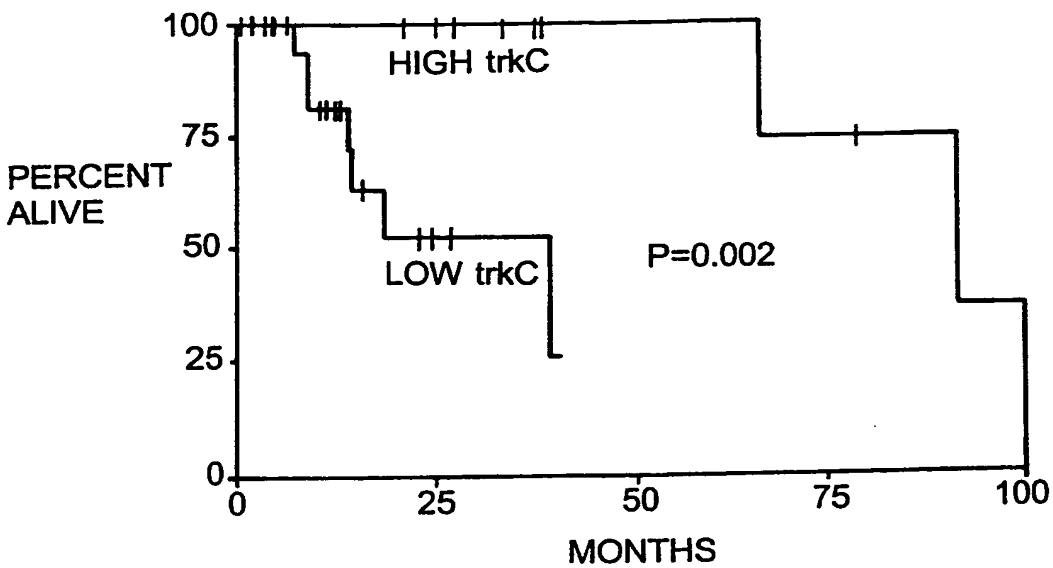
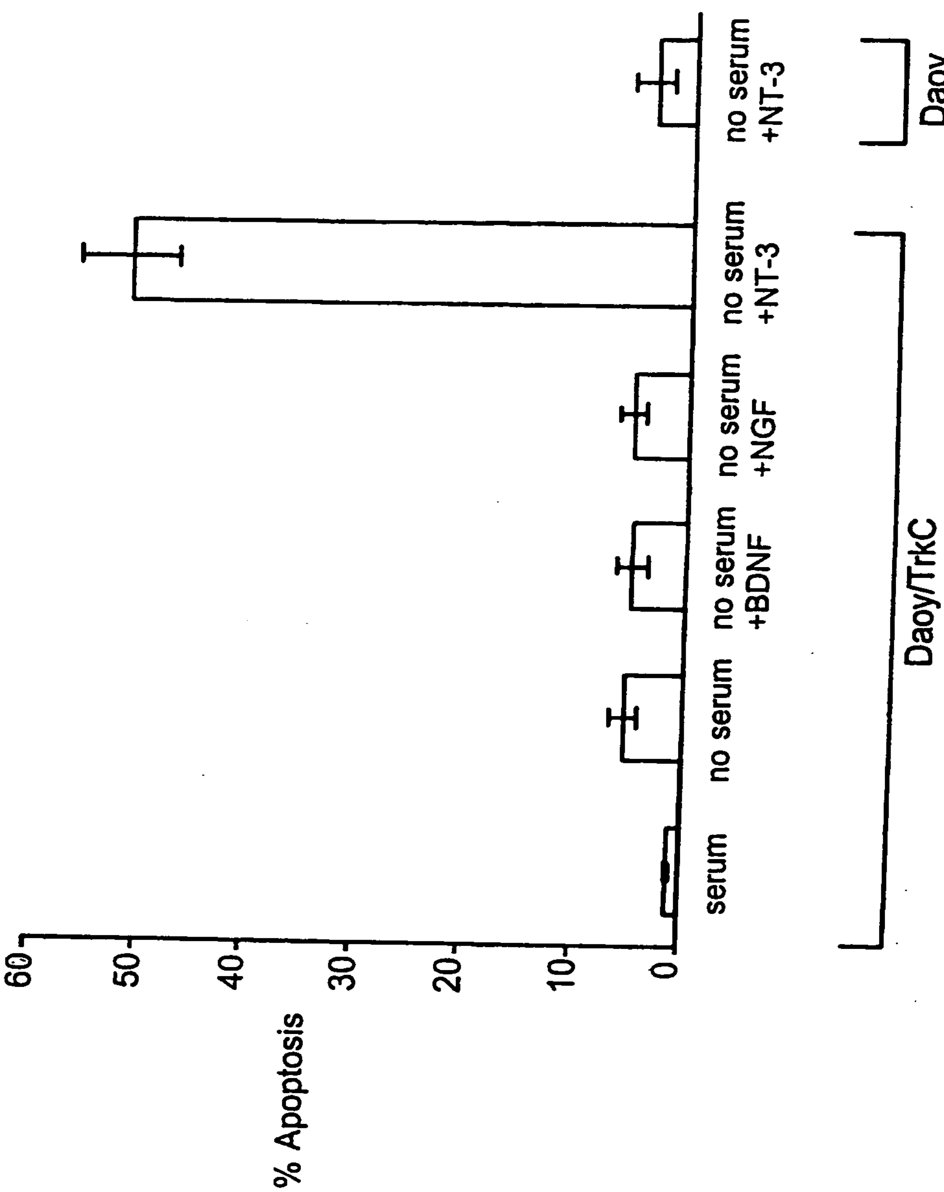

100
75
50
25
0
PERCENT
ALIVE
HIGH trkC
LOW trkC
P=0.002
0
25
50
75
100
MONTHS

FIG. 1B


% Apoptosis
60
50
40
30
20
10
0
serum
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no serum
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no serum
+NGF
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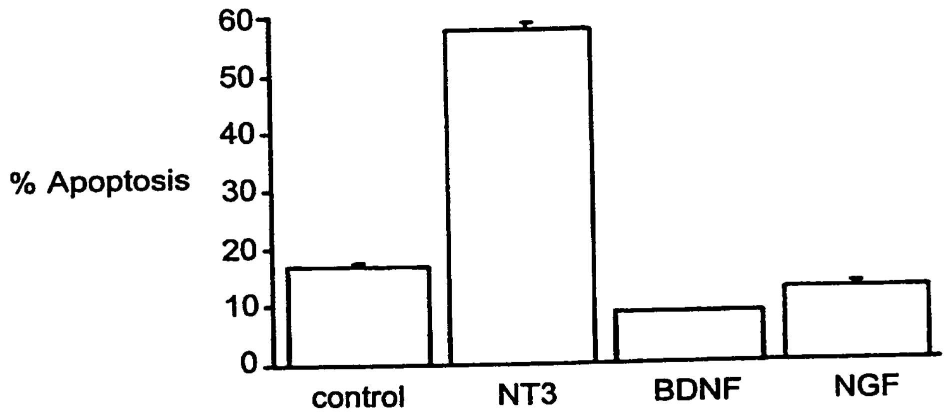
no serum
+NT-3
Daoy/TrkC
Daoy

FIG. 2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50
40
30
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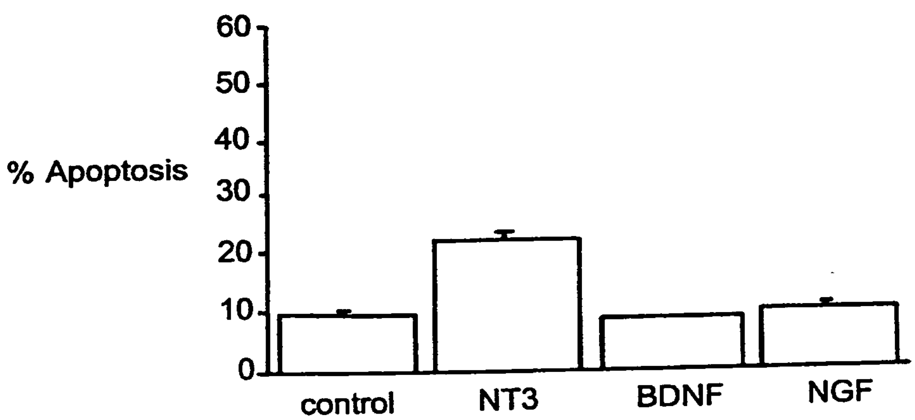
control
NT3
BDNF
NGF

FIG. 3A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control
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BDNF
NGF

FIG. 3B

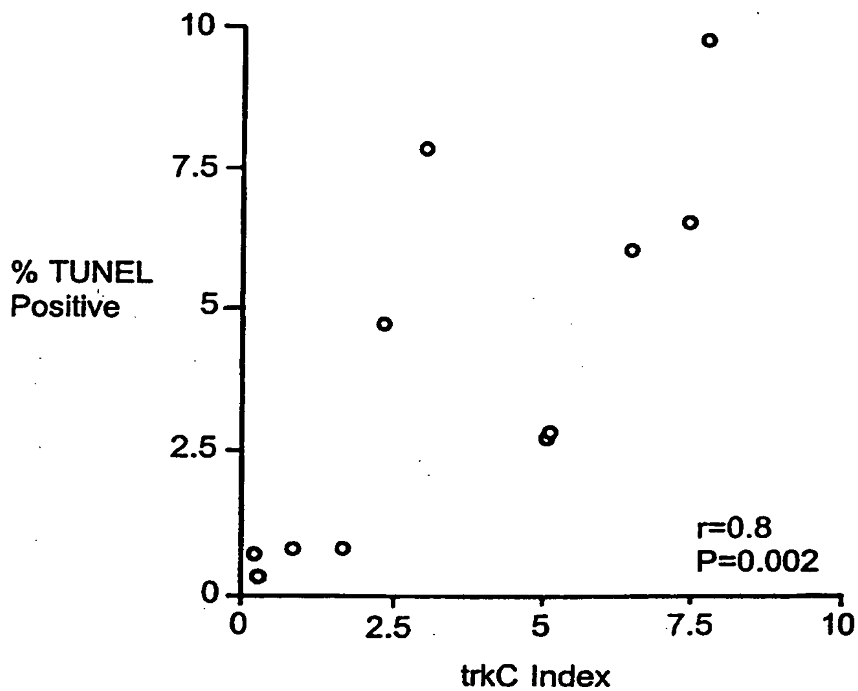

FIG. 4

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02871

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/02, 21/04; C12N/ 15/85, 15/86; C12Q 1/68; G01N 33/53
US CL : 435/6, 7.1, 325; 530/ 387.1, 399; 536/23.1, 23.5, 23.51, 24.31
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 325; 530/ 387.1, 399; 536/23.1, 23.5, 23.51, 24.31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, HCAPLUS, WPIDS
search terms: NT3 or neurotrophin 3, medulloblastoma, trkC, apoptosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	Database HCAPLUS on STN. AN 1999:110124. KIM et al. 'Activation of Neurotrophin-3 receptor TrkC induces apoptosis in medulloblastomas'. Cancer Research. 1999, Vol. 59, No. 3, pages 711-719.	1-14
Y,O	Database BIOSIS on STN. AN 1996:491025. POMEROY et al. 'Activation of neurotrophin 3 receptor induces apoptosis in a medulloblastoma cell line'. Society for Neuroscience Abstracts. 1996, Vol. 22, No 1-3, page 1000. Meeting Info 26th annual meeting of the society for Neuroscience, (Washington, D.C.) 16-21 November 1996.	1-14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 MAY 1999	25 MAY 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	GEETHA P. BANSAL JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/09292

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUTHANTHIRAN M: "Human renal allograft rejection: molecular characterization." NEPHROLOGY, DIALYSIS, TRANSPLANTATION: OFFICIAL PUBLICATION OF THE EUROPEAN DIALYSIS AND TRANSPLANT ASSOCIATION - EUROPEAN RENAL ASSOCIATION. ENGLAND 1998, vol. 13 Suppl 1, 1998, pages 21-24, XP002267088 ISSN: 0931-0509	1,5,6, 10,11
Y	the whole document	2-4,7-9, 12-14

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
16 January 2004	27.05.2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Botz, J

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INTERNATIONAL SEARCH REPORT

International Application No
EP 03/09292

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHARMA V K ET AL: "Molecular correlates of human renal allograft rejection" TRANSPLANTATION PROCEEDINGS, vol. 30, no. 5, August 1998 (1998-08), pages 2364-2366, XP002267089 Meeting on New Dimensions in Transplantation: Weaving the Future;Florence, Italy; February 16-19, 1998 ISSN: 0041-1345	1,5,6, 10,11
Y	the whole document	2-4,7-9, 12-14

X	TANG W H ET AL: "Activation of the serine proteinase system in chronic kidney rejection." TRANSPLANTATION. UNITED STATES 27 JUN 1998, vol. 65, no. 12, 27 June 1998 (1998-06-27), pages 1628-1634, XP008026499 ISSN: 0041-1337	1,5,6, 10,11
Y	the whole document	2-4,7-9, 12-14

X	STREHLAU JURGEN ET AL: "Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 94, no. 2, 1997, pages 695-700, XP002267122 1997 ISSN: 0027-8424	1,5,6, 10,11
Y	the whole document	2-4,7-9, 12-14

X	SUTHANTHIRAN MANIKKAM: "Clinical application of molecular biology: A study of allograft rejection with polymerase chain reaction" AMERICAN JOURNAL OF THE MEDICAL SCIENCES, vol. 313, no. 5, 1997, pages 264-267, XP008026444 ISSN: 0002-9629	1,5,6, 10,11
Y	page 264 - page 266; table 1	2-4,7-9, 12-14

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Form PCT/ISA/210 (continuation of second sheet) (January 2004)

International Application No
P[redacted]P 03/09292

C.(Continuation) DOCUMENTS CO[redacted]ERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DELARUE FRANCOISE ET AL: "Prognostic value of plasminogen activator inhibitor type 1 mRNA in microdissected glomeruli from transplanted kidneys" TRANSPLANTATION (BALTIMORE), vol. 72, no. 7, 15 October 2001 (2001-10-15), pages 1256-1261, XP008026393 ISSN: 0041-1337 the whole document -----	1-14
Y	KAMOUN MALEK: "Cellular and molecular parameters in human renal allograft rejection" CLINICAL BIOCHEMISTRY, vol. 34, no. 1, February 2001 (2001-02), pages 29-34, XP001176799 ISSN: 0009-9120 page 30 - page 31 -----	1-14

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